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A third human retinoic acid receptor, hRAR-γ

(skin/nuclear receptors/vitamin A/transcriptional activation)

A. KRUST, PH. KASTNER, M. PETKOVICH, A. ZELENT, AND P. CHAMBON*

Laboratoire de Génétique Moléculaire des Eucaryotes du Centre National de la Recherche Scientifique, Unité 184 de Biologie Moléculaire et de Génie Génétique de l'Institut National de la Santé et de la Recherche Médicale, Institut de Chimie Biologique, Faculté de Médecine, 11, rue Humann, 67085 Strasbourg Cédex, France

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ABSTRACT Retinoic acid receptors (RARs) are retinoic acid (RA)-inducible enhancer factors belonging to the superfamily of steroid/thyroid nuclear receptors. We have previously characterized two human RAR (hRAR-α and hRAR-β) cDNAs and have recently cloned their murine cognates (mRAR-α and mRAR-β) together with a third RAR (mRAR-γ) whose RNA was detected predominantly in skin, a well-known target for RA. mRAR-γ cDNA was used here to clone its human counterpart (hRAR-γ) from a T47D breast cancer cell cDNA library. Using a transient transfection assay in HeLa cells and a reporter gene harboring a synthetic RA responsive element, we demonstrate that hRAR-γ cDNA indeed encodes a RA-inducible transcriptional trans-activator. Interestingly, comparisons of the amino acid sequences of all six human and mouse RARs indicate that the interspecies conservation of a given member of the RAR subfamily (either α, β, or γ) is much higher than the conservation of all three receptors within a given species. These observations indicate that RAR-α, -β, and -γ may perform specific functions. We show also that hRAR-γ RNA is the predominant RAR RNA species in human skin, which suggests that hRAR-γ mediates some of the retinoid effects in this tissue.

Retinoic acid (RA) is a vitamin A (retinol) metabolite that has marked effects on growth of normal and malignant cells, pattern formation in limb development and regeneration, and fetal development (refs. 1 and 2 and refs. therein). Although the cellular RA binding protein, which is found in many retinoid target tissues, may be important in pattern formation during limb development (2), it is clearly absent from some cells known to respond to RA (ref. 3 and refs. therein). The direct effects of retinoids may in fact be mediated by the recently described nuclear RA receptors (RARs), which act as ligand-inducible transcriptional enhancer factors and belong to the nuclear receptor superfamily, which includes thyroid and steroid hormone receptors. Two forms of RAR have been characterized in human (hRAR-α and -β) (4–7) and mouse (mRAR-α and -β) (8) and a third receptor (mRAR-γ) has been recently identified in mouse (8). Studies of the distribution of mRNA for these receptors in postnatal and adult mouse tissues have indicated that, whereas RAR-α is ubiquitously expressed and RAR-β RNA is present at lower levels in a number of the tissues examined, RAR-γ RNA is found almost exclusively and at comparatively high levels in skin (8).

Given the essential role of retinoids in epidermal differentiation and their effectiveness in the treatment of several skin disorders (refs. 9–11 and refs. therein), the determination of the cellular distribution of RAR-γ and of its specific function in human skin will be of obvious biological and clinical interest. To this end, we therefore sought to clone the cDNA for the human counterpart of the mRAR-γ. We report here the characterization of the hRAR-γ cDNA and show that the corresponding mRNA is the predominant RAR RNA species in skin.

MATERIALS AND METHODS

Cloning and Sequencing of hRAR-γ cDNA. Approximately 10^6 phage from T47D cell breast cancer λgt11 (4) and λgt10 (a gift of J. M. Garnier, Laboratoire de Génétique Moléculaire des Eucaryotes, Strasbourg, France) libraries were screened with a ^{32}P-labeled nick-translated cDNA probe (4) encompassing the entire mRAR-γ open reading frame (ORF) as described (8). Eight clones were isolated that gave strong hybridization signals after two rounds of screening.

cDNA inserts were subcloned into the *Eco*RI site of pEMBL19$^+$ (4) and sequenced by the dideoxy nucleotide chain-termination technique on both strands (12) with synthetic oligonucleotide primers.†

RNA Isolation and Northern Blot Analysis. Poly(A)$^+$ RNA was isolated and electrophoresed through a 1% agarose formaldehyde gel as described (8, 13). The gels were blotted onto nitrocellulose filters (Schleicher & Schüll BA85), and hybridization and washing of the filters under stringent conditions with randomly primed ^{32}P-labeled probes derived from hRAR-α, -β, and -γ cDNA were performed as described (14). Autoradiography was performed with Kodak XAR-5 films at -80°C and intensifying screens.

Transcriptional Activation by hRAR-γ0. The entire 1.5-kilobase (kb) insert from the hRAR-γD clone was inserted into the *Eco*RI site of the eukaryotic expression vector pSG5 (15), to yield hRAR-γ0. hRAR-γ0 (500 ng) was transfected into HeLa cells along with 2 μg of the $(TRE_3)_3$-thymidine kinase (tk)-chloramphenicol acetyltransferase (CAT) reporter plasmid (8), 2 μg of a β-galactosidase-expressing plasmid pCH110 (to normalize for variations in transfection efficiency), and 15 μg of carrier DNA (BSM13$^+$) as described (4, 8). Twenty-four hours after transfection, the cells were exposed for an additional 24 hr to RA or retinol with concentrations ranging from 10 pM to 1 μM. Extracts of HeLa cells were prepared and assayed for CAT activity as described (4).

RESULTS

Cloning of hRAR-γ cDNA. When Northern blots of poly(A)$^+$ RNA prepared from the human T47D breast cancer cell line were hybridized with a ^{32}P-labeled cDNA probe

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Abbreviations: RA, retinoic acid; RAR, retinoic acid receptor; hRAR-α, -β, and -γ, human RARα, RARβ, and RARγ; mRAR-α, -β, and -γ, mouse RARα, RARβ, and RARγ; ORF, open reading frame; tk, thymidine kinase; CAT, chloramphenicol acetyltransferase.

*To whom reprint requests should be addressed.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M24857).

corresponding to the entire ORF of mRAR-γ, a 3.3-kb cross-hybridizing species could be detected that was distinct from those observed using either hRAR-α or -β (see below and data not shown). Randomly primed T47D cell cDNA libraries constructed in λgt11 and λgt10 and ^{32}P-labeled mRAR-γ cDNA were used to isolate the corresponding cDNA clones. Positive clones were further processed for sequence analysis. Two of these clones, hRAR-γA and -γD (2.0 and 1.5 kb long, respectively) contained a common major ORF that conceptually encodes a 454-amino acid long protein (M_r 50,347), exhibiting 97% homology with mRAR-γ (458 amino acid residues long) (see Fig. 1, in which the sequence of the clone hRAR-γA is presented and amino acids that differ between human and mouse are boxed). Half of these differing amino acids are located at the C-terminal end of the proteins and result from a shift in the codon reading frame arising from a single thymidilic residue insertion at position 1763 of hRAR-γA (Fig. 1). This frameshift was also found in hRAR-γD and -γE cDNA clones (see below). Note that, in contrast to mRAR-γ (8), there is no in-frame stop codon upstream of the designated methionine initiation codon of hRAR-γA (see Fig. 1) and hRAR-γD (see below; Fig. 2). This assigned position for the initiating codon for hRAR-γA and -γD is therefore only tentative and based on the overall high homology of hRAR-γ and mRAR-γ amino acid sequences (see above). It is important to note that the nucleotide sequence of hRAR-γA upstream from the initiating codon of the common ORF (positions 130–414) is 77% homologous to the corresponding 5′ untranslated region of mRAR-γ cDNA (see ref. 8).

Multiple Forms of hRAR-γ cDNA Differing in Their 5′ Regions. Although hRAR-γA and -γD cDNAs encode a common 454-amino acid long sequence, they diverge for the first 20 bases at the 5′ end of hRAR-γD cDNA (Fig. 2, underlined sequence). Note that these divergent bases would place in-frame an upstream methionine codon (boxed in Fig. 2) conceptually adding 13 amino acid residues to the common 454-amino acid sequence. Three additional hRAR-γ cDNA clones were found that are not colinear with either hRAR-γA or -γD in their 5′ regions. hRAR-γC contains a 144-base-pair insertion (underlined in Fig. 2) between the residues corresponding to positions 272 and 273 of hRAR-γA. This insertion contains a termination codon (boxed in Fig. 2) in-frame with the initiation codon of the common 454-amino acid long ORF.

The last two cDNA clones, hRAR-γB and -γE, diverge from the other clones at a point that corresponds exactly to the boundary between the two exons that separately encode the A and B regions in both hRAR-α and -β (arrowhead in Fig. 2; see ref. 5). However, these two clones are different from one another; the sequence of hRAR-γE diverges from those of all of the other forms of hRAR-γ cDNAs upstream from this point, whereas the corresponding sequence of hRAR-γB is colinear with the first 209 residues of hRAR-γA cDNA and with the first 95 residues of hRAR-γC cDNA (see Fig. 2). For

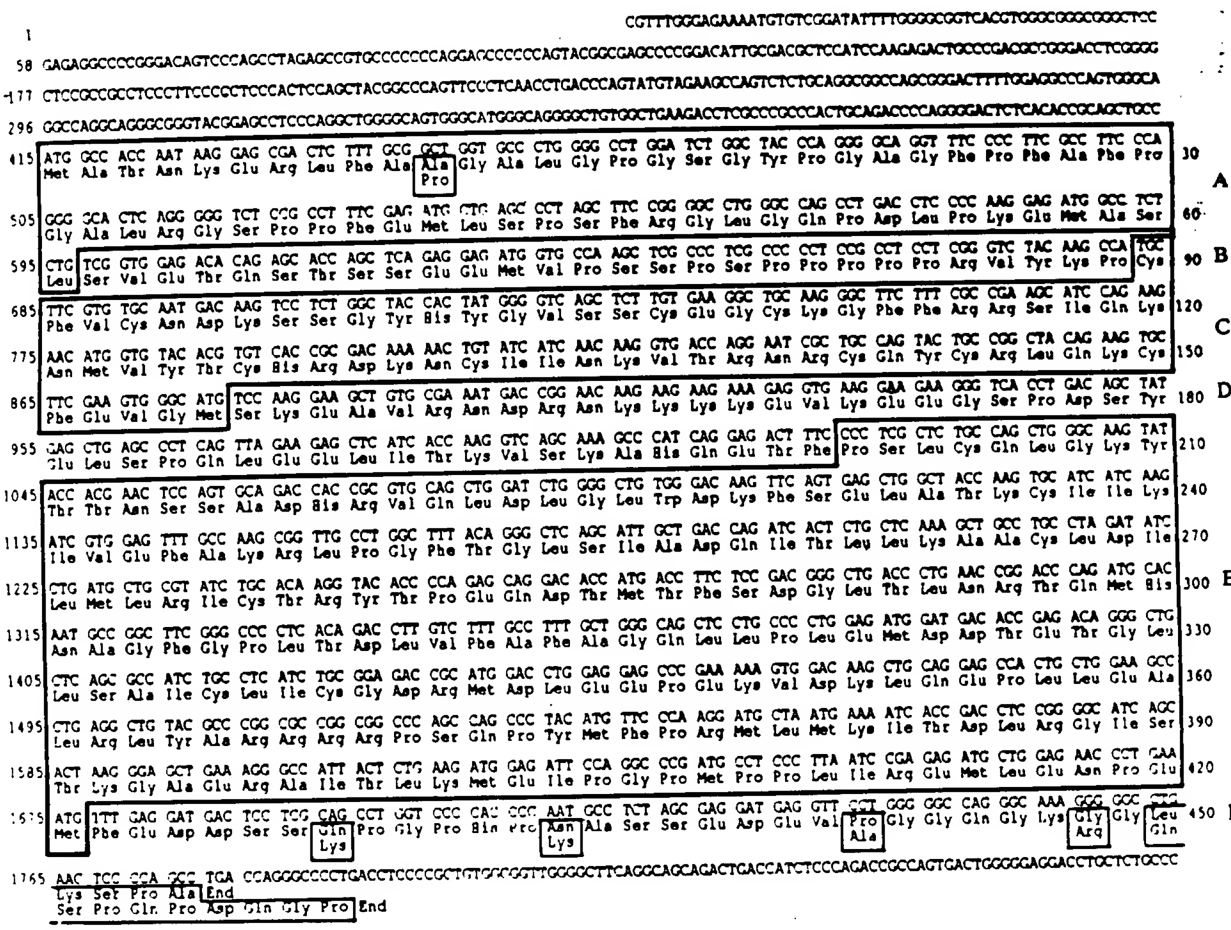

FIG. 1. hRAR-γA cDNA sequence and comparison of its deduced amino acid sequence with that of mRAR-γ. The nucleotide sequence of the hRAR-γA clone is shown from the first nucleotide following the *Eco*RI linker to nucleotide 1894. The amino acid sequence representing the ORF common to most of the clones analyzed is shown below their respective codons from the assigned initiation ATG codon (see text). The numbers on the left refer to the position of the nucleotides and those on the right refer to those of the amino acids. The sequence was divided into six regions (A–F, see text). Regions A, C, and E are boxed. Boxed amino acid residues represent those encoded by the cDNA sequence for mRAR-γ, which differ from those encoded by the hRAR-γ cDNA.

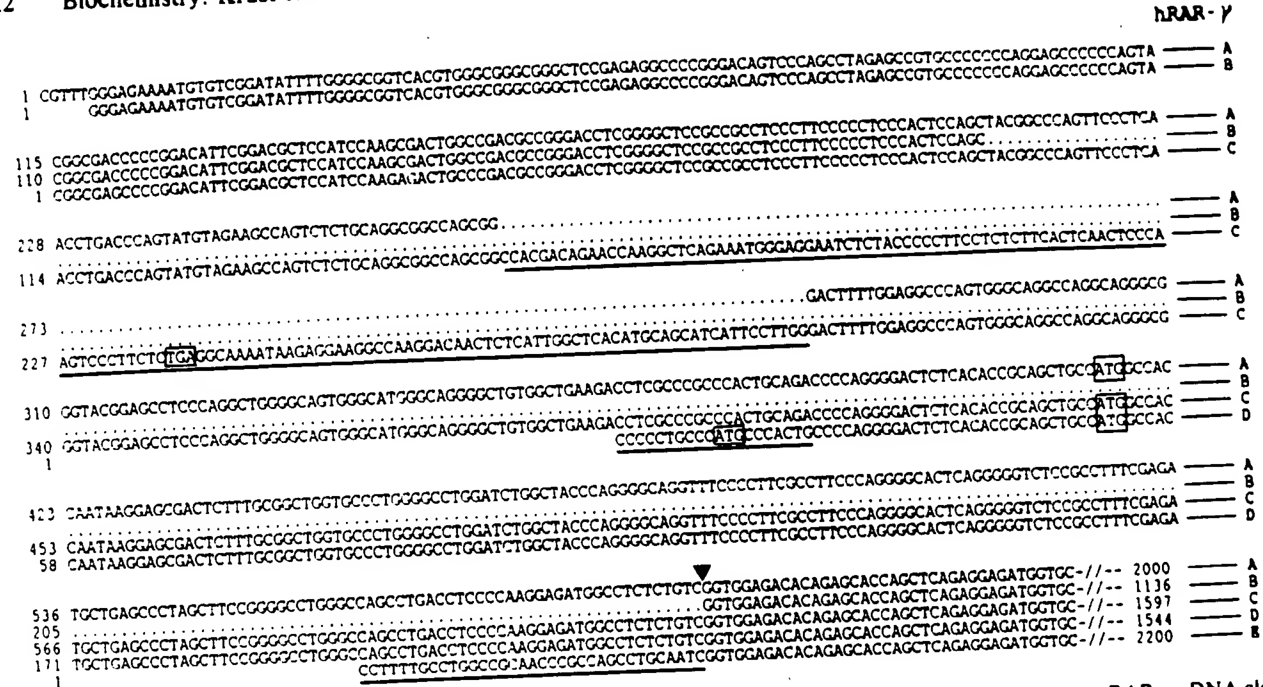

FIG. 2. Comparison of the 5′ regions of five hRAR-γ cDNAs. Alignment of the 5′ sequences of five divergent human RAR-γ cDNA clones (indicated on the right as hRAR-γ A–E) is shown. For each cDNA, the numbers on the left refer to the position of the first nucleotide in each line with respect to the most 5′ nucleotide (numbered from 1 in each case). The size of each of the hRAR-γ cDNAs is indicated following the most 3′ nucleotide. Gaps in homology are shown by dots. The nucleotides that in hRAR-γC, -γD, and -γE cDNAs are divergent from those of hRAR-γA are underlined. Arrowhead (▼) indicates the position separating regions A and B (see text). The assigned initiation ATG for the hRAR-γ ORF common to hRAR-γA, -γC, and -γD, and the upstream stop codon in-frame with this ATG in hRAR-γC are boxed, as well as the upstream in-frame ATG in hRAR-γD.

all hRAR-γ cDNAs, except for hRAR-γC (see above), the common ORF remains open to their 5′ end, which raises the possibility of the existence of several RAR-γ proteins, differing in their N-terminal region, in addition to the protein containing the common 454-amino acid long sequence defined above.

hRAR-γ Acts as a RA-Inducible Transcription Factor. To test the ability of hRAR-γ to activate transcription, hRAR-γD cDNA was subcloned into the eukaryotic expression vector pSG5 (15) to give hRAR-γ0, which was cotransfected into HeLa cells along with a reporter plasmid, $(TRE_3)_3$-tk-CAT, which contains a synthetic RA responsive element (see ref. 8). The transfected cells were exposed to increasing concentrations of RA, and RA-induced activation of transcription was estimated 24 hr after transfection by determining the CAT activity expressed from the reporter gene (Fig. 3). Maximal transactivation was achieved at 10^{-8}–10^{-7} M RA, within a RA concentration range similar to that required to

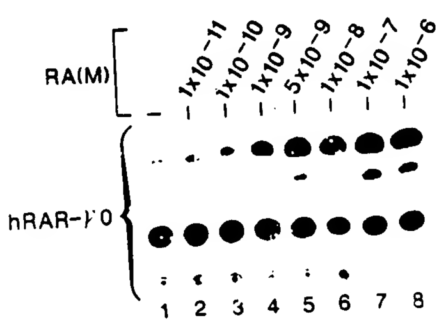

FIG. 3. RA-dependent transcriptional activation by hRAR-γO. The CAT activity resulting from activation of the reporter gene $(TRE_3)_3$-tk-CAT by hRAR-γO in the presence of RA (as indicated) is shown. Extracts of HeLa cells transiently transfected and treated with RA were assayed for CAT activity (4).

achieve maximal stimulation with expression vectors derived from hRAR-α and -β (4, 5, 8), indicating that the cloned hRAR-γ cDNAs encode a functional RAR. As previously reported for hRAR-α and -β (4, 5), retinol was a much less potent activator than either all-*trans*- or 13-*cis*-retinoic acid (data not shown).

hRAR-γ RNA Is the Predominant RAR RNA Species in Skin. In a recent study (8), we have compared the distribution of mRAR-α, -β, and -γ mRNA in a variety of adult mouse tissues and found that mRAR-α and -β were expressed in many of the tissues examined, whereas the expression of mRAR-γ RNA was almost exclusively restricted to skin (with very low levels of expression in lung and spleen). Northern blot analyses of total RNA extracted from adult human skin and poly(A)$^+$ RNA prepared from fetal skin (Fig. 4, lanes 5 and 6, respectively), using ^{32}P-labeled hRAR-α, -β, and -γ cDNA probes under stringent conditions, showed that hRAR-γ RNA was also the predominant RAR RNA species in both adult and fetal human skin (the densitometric scanning of the reference actin RNA signal indicated that there was 7-fold more actin mRNA in lane 6 than in lane 5). hRAR-γ RNA may also be present in human lung (lane 7), albeit at a much lower level.

We also examined the expression of all three RAR RNAs in several human cell lines, including the breast cancer cell line T47D from which hRAR-α and -γ cDNAs have been cloned (lane 8). A comparatively high level of hRAR-γ RNA was seen in a human teratocarcinoma cell line (lane 2), which is reminiscent of a similar predominance of mRAR-γ RNA in the mouse F9 teratocarcinoma cell line (8). In contrast, we could not detect expression of either hRAR-γ or -β in the HepG2 human hepatoma cell line (lane 3), and the relative level of hRAR-γ was low in both the adenovirus-transformed 293 cell line (lane 3) and a neuroblastoma cell line (lane 1). Note also that hRAR-α appears to be ubiquitously expressed and that the ratio of the two subspecies of hRAR-α and -β

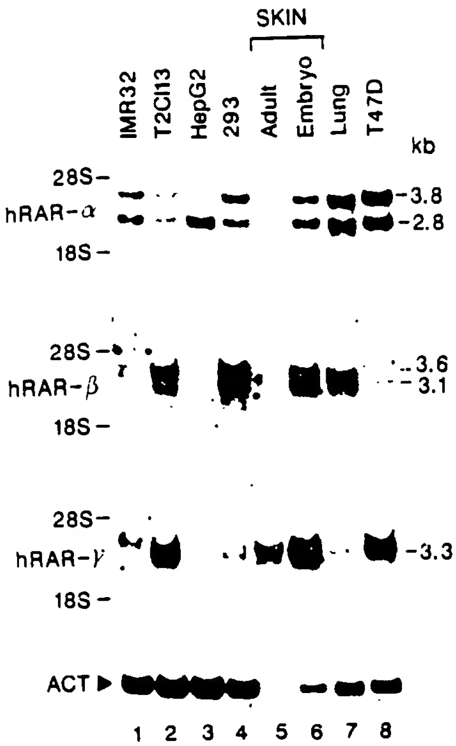

FIG. 4. Northern blot analysis of hRAR-α, -β, and -γ RNAs from various tissues and cells. RNA preparation, blotting, electrophoresis, and hybridization with ^{32}P-labeled cDNA probes were as described in *Materials and Methods*. Lanes: 1–4, 7 μg each of poly(A)$^+$ RNA prepared from human neuroblastoma cells IMR32 (16), human teratocarcinoma cells T2Cl13 (17), human hepatoma cells HepG2 (18), and human adenovirus-transformed cells 293 (19) as indicated; 5, 20 μg of total RNA extracted from adult human skin; 6 and 7, 4 μg each of poly(A)$^+$ RNA from human fetal skin and adult lung as indicated; 8, 4 μg of poly(A)$^+$ RNA from T47D breast cancer cells. Autoradiography was for 14 hr, 7 days, and 48 hr with hRAR-α, -β, and -γ cDNA probes, respectively. Blots were rehybridized with an actin (ACT) cDNA probe (8) to check the integrity of the RNA preparation. Sizes shown on the right in kb for the RAR RNA species were calculated from their migration relative to the 28S and 18S rRNAs whose sizes are 4712 and 1869 nucleotides, respectively (20–22).

RNA was variable among the different cell lines examined. The significance of these differences in size and abundance of hRAR-α and -β transcripts remains to be established.

The Cognate Members of Human and Mouse RARs Are Highly Conserved. The structure of nuclear receptors was divided into six discrete regions designated A–F, originally defined by amino acid sequence comparisons between various members of the family and by analyzing the conservation across species of the primary structure of a given member of the nuclear receptor family (see ref. 23 and refs. therein). The three human RARs can be similarly divided (see ref. 5 and Fig. 1) and aligned with their murine cognates (Fig. 1; see figure 1 *a* and *b* in ref. 8). In all three cases, the comparison of the human and mouse sequences of a given RAR indicates a high degree of conservation between each of the corresponding domains (Table 1, columns 2, 3, and 4). Greater than 90% amino acid identity is apparent between each of the corresponding regions (A–F) of the human and murine homologs, except for region F of RAR-γ (column 2, row F). The lower degree of conservation (58%) between regions F of mouse and human is due to a frameshift mutation in the sequence corresponding to the end of region F (see above). A similar comparison, but between the three human RARs (columns 5, 6, and 7), reveals a high degree of homology

Table 1. Amino acid sequence similarities between human and mouse RARs

	hγ/mγ	hα/mα	hβ/mβ	hγ/hα	hγ/hβ	hα/hβ	hER/mER
A	98	98	94	25	<15	<15	95
B	100	100	100	75	86	79	80
C	100	98	100	97	94	97	100
D	100	98	98	72	62	74	80
E	100	99	99	84	90	90	96
F	58	90	92	20	<15	20	60

The amino acid sequences of regions A–F (column 1) previously defined for hRAR-α and -β (5), for mRAR-α, -β, and -γ (see ref. 8 and Fig. 1), and for hRAR-γ were compared. For each set of comparisons, the percentage of amino acid identity (after optimal alignment) is shown (columns 2–7). Column 8 corresponds to a similar comparison between regions A–F of the human (24) and mouse (25) estrogen receptors (hER and mER). hα, -β, and -γ, and mα, -β, and -γ correspond to hRAR-α, -β, and -γ, and mRAR-α, -β, and -γ, respectively.

between hRAR-α, -β, and -γ only for the regions corresponding to the DNA binding (region C) and the RA binding (region E) domains. Most noteworthy is the almost complete divergence of regions A (compare in row A, columns 5–7 with columns 2–4) and F (compare in row F, columns 5–7 with columns 2–4). The overall conservation of region D is also lower between the three human RARs than between two corresponding members of the human and mouse subfamilies (compare columns 5–7 with columns 2–4). In fact, as observed also in the case of the three mouse RARs (8), the homology is even lower for a 24-amino acid long central section of region D (≈40% sequence similarity between all three human RARs).

DISCUSSION

We have described here cDNA clones isolated from a human T47D cell library that contain a common ORF encoding a protein highly homologous to the recently characterized mRAR-γ (8). That this ORF encodes a functional RAR was established by its expression in HeLa cells, where it could activate the transcription of a RA-responsive reporter gene within a range of RA concentration similar to that reported for transcriptional trans-activation by the different members of the human (4, 5) and mouse (8) RAR subfamily. This brings to three the number of hRARs characterized to date.

The extensive amino acid sequence homology between hRAR-γ and mRAR-γ suggests strongly that they are functionally equivalent. In this respect, we note that the hRAR-γ gene is located on chromosome 12, whereas the mRAR-γ gene is located on the mouse chromosome counterpart, chromosome 15 (M. G. Mattei, M.P., E. Passage, A.Z., Ph.K., A.K., and P.C., unpublished data). The comparisons of the amino acid sequences of human and mouse RARs also suggest strongly that hRAR-α and mRAR-α, and hRAR-β and mRAR-β, are functionally equivalent. It is particularly striking that the A, B, D, and F regions are highly conserved between human and mouse for a given member of the RAR subfamily, whereas they are not, or to a much lesser extent, when comparing the different RARs of either human or mouse (Table 1). These regions are usually not as well conserved across species for a given steroid hormone receptor (see Table 1, column 8, for a comparison between the human and mouse estrogen receptors). The almost complete conservation across species in the case of each of the three members of the RAR subfamily indicates that these regions must be important for their functional specificity. Since the DNA-binding domains (regions C) of RAR-α, -β, and -γ are highly conserved, all three receptors may interact with the same responsive elements. The A/B regions of the estrogen and progesterone receptors appear to play a role in specific

transcriptional trans-activation of some target genes (refs. 23 and 26 and refs. therein). The different A/B regions of the three RARs may have a similar function. The lower conservation for the F region of human and mouse RAR-γ is due to a single base difference in the region encoding the last amino acids of this region. The same single base difference was found in two independent mRAR-γ cDNA clones (8) and in all three hRAR-γ cDNA clones that contain this region (see above). Moreover, we can exclude the possibility that the T47D breast cancer cell hRAR-γ gene bears a mutation not present in normal cells, since the same sequence was found in cDNA derived from skin hRAR-γ mRNA and amplified by polymerase chain reaction (data not shown). Several hRAR-γ cDNA clones that we have analyzed here differ in their 5′ regions. In two cases (hRAR-γB and -γE), these differences may well reflect alternative splicing, since the point of divergence is located at a position that is known to correspond to the boundary between the exons separately encoding regions A and B of both hRAR-α (N. Brand and P.C., unpublished results) and hRAR-β (see refs. 4, 5, and 27). We note that a similar situation may exist in the case of the thyroid hormone receptors for which two β forms diverging at the corresponding exon–intron boundary (4, 28) have been recently described (29). Whether alternative splicing is also responsible for the other differences seen in the hRAR cDNA 5′ regions is unknown. Since the hRAR-γ major ORF remains open up to the 5′ end in four of five cDNA clones that we have sequenced here, it is possible that there are several hRAR-γ proteins that differ in their N-terminal sequence and thus exhibit different target gene specificity (see above).

We have shown elsewhere that mouse RAR-γ RNA is expressed at a much higher level in skin than in any other adult tissue analyzed (8). hRAR-γ RNA is also the predominant RAR RNA species in human adult and fetal skin (Fig. 4). This observation is particularly interesting in view of the effect of retinoids on skin, both in normal and pathological states (see Introduction for refs.). That hRAR-γ RNA is the predominant RAR species in a human teratocarcinoma (Fig. 4) suggests that hRAR-γ, along with hRAR-α and -β, may also play a role during embryogenesis.

It has been thought that the range and diversity of effects of RA would preclude the possibility that a single molecular mechanism might account for all of them. Direct control of gene expression by multiple RARs may, however, account for a large proportion of the RA effects, provided that the various RARs would exhibit different spatial and temporal patterns of expression and would specifically activate different target genes.

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